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Aquatic Toxicology 82 (2007) 215–226

AQUATIC TOXICOLOGY

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Identification of gene expression indicators for thyroid axis disruption in a *Xenopus laevis* metamorphosis screening assay Part 2. Effects on the tail and hindlimb

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Received 19 December 2006; received in revised form 1 February 2007; accepted 3 February 2007

Abstract

Thyroid hormones (TH), thyroxine (T_4) and 3,5,3′-triiodothyronine (T_3), play crucial roles in regulation of growth, development and metabolism in vertebrates and are targets for endocrine disruptive agents. Perturbations in TH action can contribute to the development of disease states and the US Environmental Protection Agency is developing a high throughput screen using TH-dependent metamorphosis of the *Xenopus laevis* tadpole as an assay platform. Currently this methodology relies on external morphological endpoints and changes in central thyroid axis parameters. However, exposure-related changes in gene expression in TH-sensitive tissue types that occur over shorter time frames have the potential to augment this screen. Using a combination of cDNA array and real time quantitative polymerase chain reaction (QPCR) analyses, this study identifies molecular markers in tissues peripheral to the central thyroid axis. We examine the hindlimb and tail of tadpoles up to 96 h of continuous exposure to T_3, T_4, methimazole, propylthiouracil, or perchlorate. Several novel biomarker candidates are indicated that include transcripts encoding importin, RNA helicase II/Gu, and defender against death protein, DAD1. In combination with previously-identified biomarker candidates, these transcripts will greatly augment the predictive and diagnostic power of the *Xenopus* metamorphosis assay for perturbation of TH action.

Keywords: cDNA array; Quantitative real time polymerase chain reaction; Amphibian; Frog; Metamorphosis; Tail; Hindlimb

1. Introduction

As part of the mandate of the Food Quality Protection and Safe Drinking Water Acts established by the US Congress in 1996 and in response to the recommendations of the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC), the US Environmental Protection Agency (US EPA) is developing and implementing a screening approach for thyroid axis disruption using metamorphosis in the *Xenopus laevis* frog tadpole (EPA, 1998). This assay takes advantage of the dependence of amphibian metamorphosis on normal thyroid function and the use of whole animals to allow consideration of physiology, chemical biotransformation, and cellular context.

Gene expression monitoring is one screening approach that has the potential to serve as a reliable predictor of whole organism effects and specific mechanisms of action of a particular chemical, class of chemicals or mixture of unrelated chemicals. Since changes in gene expression often precede overt morphological and physiological changes, development of molecular endpoints for incorporation into the existing methodology also has the potential to reduce assay duration. To date, changes in morphology of early prometamorphic *X. laevis* tadpoles have been the primary endpoints in the context of a metamorphosis assay (Degitz et al., 2005; Tietge et al., 2005). However, standard toxicological approaches that focus on tissue and organismal effects fall short in adequately discerning sublethal molecular mechanisms of action.

Metamorphosis involves the transition of the larval tadpole to a juvenile frog. The dramatic structural and functional changes of larval tissues during this developmental process are completely dependent upon thyroid hormones (THs) (Atkinson et al., 1996; Damjanovski et al., 2000; Shi, 2000). As in mam-

DOI of original article:10.1016/j.aquatox.2007.02.013.
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doi:10.1016/j.aquatox.2007.02.014

mals, thyroxine (T_4) is the main secretory product of the amphibian thyroid gland (Buscaglia et al., 1985) and it is converted by deiodinase activity in peripheral tissues to the more active 3,5,3′-triiodothyronine (T_3) form (Becker et al., 1997; Huang et al., 1999; Kawahara et al., 1999). T_3 functions primarily by regulating gene transcription through high affinity binding to specific nuclear TH receptors (TRs) that interact with T_3 responsive elements (TREs) located within the promoters of target genes (Sap et al., 1986; Weinberger et al., 1986).

Metamorphosis has three distinct developmental phases: premetamorphosis, prometamorphosis, and metamorphic climax. During premetamorphosis, the tadpole is competent to respond to exogenous TH (which can induce precocious metamorphosis) but is functionally athyroid. Prometamorphosis begins with maturation of the thyroid gland and low-level secretion of TH that initiates the first metamorphic changes such as limb growth. TH levels continue to rise and peak dramatically at metamorphic climax, which is characterized by the rapid, overt remodeling of the tadpole. Different tissues respond to TH in different ways. For example, the tail regresses, the brain remodels and the limbs grow and differentiate. The mechanisms whereby tissue response specificity is determined are not fully understood but TR-mediated changes in gene expression play pivotal roles. Therefore, an important aspect in the development of gene expression-based biomarkers is to determine tissue sensitivities to model compounds and to establish the appropriate time point(s) for assessment.

To this end, we exposed early prometamorphic tadpoles to various concentrations of T_3, T_4, and the TH synthesis inhibitors: methimazole (MMI), propylthiouracil (PTU), and perchlorate (PER). MMI and PTU inhibit TH production by blocking the formation of active iodide *via* the peroxidase system (Cooper et al., 1984) and PER ions are competitive inhibitors of iodide uptake (Wolff, 1998). However, all three may have direct effects on peripheral tissues as well. MMI, PTU, and PER have been used to therapeutically treat hyperthyroidism, but PER is also a persistent environmental contaminant (Soldin et al., 2001) and is detected in groundwater at concentrations ranging from 200 μg/L to 3700 mg/L in highly contaminated sites (Urbansky, 1998).

The tadpole hindlimb responds rapidly to increasing levels of endogenous THs during natural metamorphosis by growth and remodeling and is regarded as a highly TH-sensitive tissue (Buckbinder and Brown, 1992). In contrast, the tail is morphologically less sensitive to TH presence and does not regress until late in the metamorphic program when endogenous TH levels are high (Shi, 2000). However, in the context of mRNA expression, we determined that TRα, TRβ and basic transcription element binding protein (BTEB) transcript levels were considerably more elevated in the tail upon exposure to low concentrations of THs compared to the hindlimb (Zhang et al., 2006). Neither the tail nor the hindlimb showed a marked alteration in the levels of these transcripts upon exposure to MMI, PTU, or PER (Zhang et al., 2006). We have also demonstrated that the brain showed the greatest changes in gene expression within 96 h of exposure to T_4, MMI, PTU, and PER whereas exposure to T_3 at the concentrations tested did not have a great effect (Helbing et al., 2007; Zhang et al., 2006). Together these data accentuate the need and importance of analyzing multiple tissues in the establishment of robust molecular endpoints that can effectively identify exposure-related effects during the *Xenopus* metamorphosis assay.

In this study, we define elements of the gene expression program in the hindlimb and tail by cDNA array and QPCR analyses that are altered following exposure to T_3, T_4, MMI, PTU, or PER. We identify a number of gene transcripts that are affected by exposure to these chemicals and present a discrete subset of gene transcripts that have strong potential for use as gene expression indicators of thyroid axis disruption within 96 h of continuous chemical exposure.

2. Animals, materials and methods

2.1. Experimental animals

X. laevis tadpoles used in this study were obtained from an in-house culture and exposures were done at the US Environmental Protection Agency, Mid-Continent Ecology Division in Duluth. Details about animal husbandry and maintenance were as described previously (Zhang et al., 2006). All protocols were reviewed and approved by the Mid-Continent Ecology Division animal use and care committee.

2.2. Water characteristics

Lake Superior water (LSW) used for all tests was filtered through sand, a 5-μm filter, a 0.45-5-μm filter, sterilized with ultraviolet light and heated to the appropriate test tank temperature of $20.9 \pm 0.2\,^{\circ}C$ ($n = 588$). Exposure tanks were immersed in a water bath system (water bath temperatures were continuously monitored) to maintain temperature uniformity between tanks. Dissolved oxygen (DO) was measured weekly during all tests using a YSI Model 550A DO meter (Fisher Scientific, Hanover Park, IL) that was calibrated prior to use by the air saturation method. The range of DO measurements across all studies was 6.29–7.80 mg/L. All other water characteristics were measured using methods described by the American Public Health Association (APHA, 1992). The range of pH readings conducted weekly on a minimum of 12 tanks during all tests across all studies was 7.65–8.06. Hardness and alkalinity determinations were made once during each study on a minimum of three tanks including one control and two treatment tanks. The range for total hardness was 47.0–47.5 mg/L $CaCO_3$. The range for all alkalinity measurements was 39.5–40.0 mg/L $CaCO_3$.

2.3. Chemicals

T_4, T_3, methimazole (MMI), 6-propylthiouracil (PTU) and sodium perchlorate (PER) were obtained from Sigma (St. Louis, MO). A stock solution of T_3 was prepared by dissolving 29 mg T_3 into 100 ml of 50 mM NaOH. A diluted stock solution of T_3 (234 nM) was prepared by addition of 10 ml T_3 stock to 19 L of

LSW. T_4 stock solutions (5.16 μM) were prepared by dissolving 72.2 mg T_4 in 18 L of LSW. Separate stock solutions for MMI (1250 mg/L) and PER (492 mg/L) were made in a 19 L glass carboy by dissolving each chemical in LSW using a stir plate and a magnetic stir bar. Stock solutions for PTU (600 mg/L) were made in a 19 L glass carboy using a high speed top stirrer to dissolve the chemical in LSW that had been previously heated to 40 °C.

2.4. Exposure system

A computerized exposure system was used for all studies. This flow-through system, whose components are glass, stainless steel, and Teflon, generated five duplicated exposure concentrations for each chemical with a dilution factor of two for T_4 and T_3, as well as duplicate controls. Exposure tanks were glass aquaria (22.5 cm × 14.0 cm × 16.5 cm deep) equipped with 13 cm standpipes, which resulted in an actual tank volume of 4.0 L. The flow rate to each tank was 25 ml/min. Fluorescent lamps provided a photoperiod of 12 h:12 h light:dark at an intensity that ranged from 61 to 139 lumens at the water surface.

2.5. Animal exposures

2.5.1. Experiment 1 (T_4 and T_3)

Twenty-eight early prometamorphic (NF stage 54; Nieuwkoop and Faber, 1956; Shi, 2000) tadpoles were continuously exposed to three separate T_4 concentrations (10, 20.1, and 40.3 nM), and a LSW control in one exposure set, or five different T_3 concentrations (0.48, 0.97, 1.92, 3.84, and 7.68 nM), and a LSW control in the second exposure set as described in detail in Zhang et al. (2006). Each chemical exposure concentration was replicated twice along with the associated LSW control. At 24, 48, and 96 h two animals per exposure replicate (four animals total per each individual treatment) were randomly selected, euthanized in MS-222, and preserved in RNA*later* (Ambion Inc., Austin, TX, USA) for analysis of gene expression. On exposure day 14, all remaining organisms were euthanized in MS-222, weighed, and developmentally staged in a blind evaluation. Animals exposed to either chemical showed an acceleration of metamorphosis which was published previously (Zhang et al., 2006).

2.5.2. Experiment 2 (MMI, PTU and PER)

NF stage 54 tadpoles were continuously exposed to a single concentration of PTU (20 mg/L), MMI (100 mg/L) or PER (4 mg/L). We have previously shown that exposure to these concentrations resulted in an increase in thyroid gland size at day 8 and significantly delayed metamorphosis at 14 days (Degitz et al., 2005; Tietge et al., 2005). The exposure regimen details are recorded elsewhere (Zhang et al., 2006). Briefly, tadpoles were randomly placed into 24 tanks (20 tadpoles/tank) and exposed (six tanks/chemical) to a single concentration of each chemical. At 24, 48, and 96 h five tadpoles from two of the six tanks (10 tadpoles per each individual treatment) were randomly selected, euthanized in MS-222, and preserved in RNA*later* for analysis of gene expression.

2.6. Chemical analysis

Water samples collected from all of the exposure sets were measured for the actual concentrations of the chemical additives as described in (Degitz et al., 2005; Tietge et al., 2005). All standard deviations were within 10% of the nominal concentrations. Therefore, the nominal concentrations are reported.

2.7. Isolation of total RNA

Tails and hindlimbs were collected from each individual tadpole and total RNA was isolated using TRIzol reagent as described by the manufacturer (Invitrogen Canada Inc., Burlington, Ontario, Canada). Mechanical disruption of each tissue utilized 300 μl (hindlimbs) or 700 μl (tail) TRIzol reagent, a 3 mm diameter tungsten-carbide bead, and safe-lock Eppendorf 1.5 ml microcentrifuge tubes in a Retsch MM301 Mixer Mill (Fisher Scientific Ltd., Ottawa, ON) at 20 Hz for 6 min. Mixing chambers were rotated 180° halfway through the homogenization procedure. Twenty micrograms of glycogen (Roche Diagnostics, Laval, PQ) was added prior to isopropanol precipitation to maximize RNA yield. Isolated RNA was subsequently resuspended in 20 μl (hindlimb) or 40 μl (tail) diethyl pyrocarbonate (DEPC)-treated water and stored at −70 °C.

2.8. Gene expression profiling

Amplified RNA (aRNA) was produced using the MessageAmp aRNA Kit as per the manufacturer's protocol (Ambion) from 1 μg total RNA from individual animals of a subset of the exposures tested. We focused on 48 h for the tail and 24 h for the hindlimb based upon previous work indicating that these time points were appropriate for examining gene expression changes (Zhang et al., 2006). For the THs, we initially examined tadpole tissues exposed to either 1.92 nM T_3 or 10 nM T_4 ($n = 4$ for each) with arrays and compared the expression profiles to those obtained from time- and experiment-matched controls ($n = 3$ each). These concentrations evoke equivalent responses in the acceleration of metamorphosis and in the induction of TRβ and BTEB transcripts in the tail (Zhang et al., 2006). For MMI, PTU, and PER, tissues from three individuals were examined for each chemical tested along with time- and experiment-matched controls. For this experimental set, we examined both 24 and 96 h time points. Once candidate gene transcripts were identified, we then performed QPCR analysis on these and additional concentrations on a gene-by-gene basis.

Five hundred nanograms of aRNA were annealed with 500 ng random hexamer oligonucleotide (Amersham Biosciences Inc., Baie d'Urfé, QC, Canada), and cDNA was prepared using MMLV RNase H^- Superscript II reverse transcriptase as described by the manufacturer (Invitrogen) with the following modifications: the dNTP mix consisted of 500 μM each of dGTP, dTTP, and dCTP, 4 μM dATP, and 50 μCi [α-^{32}P] dATP (Perkin-Elmer Life Sciences, Inc., Boston, MA, USA). RNA was removed from the radiolabeled cDNA by addition of

10 μl 1 M NaOH and incubation at 70 °C for 10 min. Samples were cooled to room temperature and 10 μl of 1 M HCl were added to neutralize the reactions. The radiolabeled cDNA was purified using a QIAquick PCR purification kit (Qiagen, Mississauga, ON) and, immediately prior to hybridization, was heat denatured for 5 min at 95 °C and then quickly cooled on ice for 5 min.

The frog MAGEX cDNA array was purchased from ViagenX Biotech Inc. (Victoria, BC, Canada) and is described in detail elsewhere (Crump et al., 2002). Briefly, the array consisted of 401 cDNA fragments of ~500 bp in length. Each fragment was amplified using gene sequence-specific primers and spotted in duplicate at adjacent grid positions on a nylon membrane support. Approximately 90% of the gene sequences present on the array originated from *X. laevis*, while the remainder were isolated from *R. catesbeiana*. Each membrane contained two intron controls to monitor for genomic DNA contamination. Details of the array platform and the raw and normalized data are available from the Gene Expression Omnibus (Accession numbers GPL4801 and GSE7051 National Center for Biotechnology Information, National Institutes of Health). Prehybridization, hybridization, and posthybridization washes were performed at 65 °C. Hybridizations were carried out in 20 ml of hybridization solution containing 4× SSC, 10% (w/v) dextran sulfate, 1.0% (w/v) SDS, and 0.5% (w/v) Blotto. Prewarmed hybridization solution was added to each hybridization tube (35 mm i.d. × 150 mm length; Amersham) containing the array membrane and prehybridization was allowed to continue for 2 h. To standardize subsequent exposure times to the phosphorimager screens, equal counts of radiolabeled cDNA samples (final concentration of 5×10^5 cpm/ml) were then added and allowed to hybridize for 18 h. After hybridization, the membranes were rinsed briefly with 50 ml 2× SSC, then washed twice with 50 ml 2× SSC/0.1% SDS for 15 min, once with 50 ml 0.1× SSC/1.0% SDS for 25 min, and rinsed with 50 ml 0.1 × SSC. The arrays were placed on 3 MM filter paper (Rose Scientific Ltd., Edmonton, AB, Canada) soaked with ddH_20 and wrapped with plastic wrap. Each processed membrane was exposed to a phosphorimager screen (Molecular Dynamics Inc., Sunnyvale, CA, USA) for 5 days. Hybridization signals were collected using a Storm 820 Gel and Blot Imaging System (Amersham) at 50 μm resolution. The resulting image data were converted to a standard 8-bit TIFF file using Photoshop Version 5.0 (Adobe Systems Inc., San Jose, CA, USA). Both non-auto- and auto-level images were prepared for analysis in order to account for signal saturation. Non-auto-leveled images provide a linear range of strong signal intensities (such as β-actin) while auto-leveled images allow for analysis of the remaining signals.

Relative expression for each gene target was collected from the image data using ImaGene Version 5.6.1 (BioDiscovery Inc., El Segundo, CA, USA). Signal intensities for each gene and blank position were determined from the median spot pixel intensities and corrected by subtracting the local median background pixel intensities. Signal intensities that were derived from areas of non-specific hybridization on the arrays were not included in the final analysis. A non-signal background was determined from the median intensity value plus one standard deviation of blank positions across the auto-level data set, and signal intensities for gene positions exhibiting values below the non signal were adjusted to this value. Saturated gene positions identified in auto-level data were replaced across all data sets by the corresponding values obtained in the non-auto-level analysis.

Data for the treatment sets were analyzed by time point. Both non-auto- and auto-leveled data for each array were normalized using a geometric mean derived from the median signal intensities of the following genes: ribosomal proteins L8, L32, and S10, GAPDH, ferritin, NM23/dinucleotide phosphate kinase, skeletal α-actin, cytoplasmic β-actin, and elongation factor-1 α chain. The choice of which of these transcripts were used for normalization within a time point was dictated by spot quality and consistency (see Section 2.10 below). The gene transcripts used for normalization within a given time point are indicated in Supplementary Table 1. After normalization, relative expression values for each gene were determined from the median of signal intensities across the replicate array membranes for each treatment time point. Genes with less than four signal intensity values were not included in further analyses. We considered a two-fold change relative to control treatments as significant in the array experiments based on the detection limitations of the cDNA array analyses.

2.9. Real-time quantitative polymerase chain reaction (QPCR)

One microgram of total RNA from the tail and hindlimbs of individual tadpoles ($n = 4$ for Experiment 1 and $n = 10$ for Experiment 2) was annealed with 500 ng random hexamer oligonucleotide (Amersham Biosciences Inc.) and cDNA was synthesized using Superscript II RNase H^- reverse transcriptase as described by the manufacturer (Invitrogen). The cDNA products were diluted 20-fold prior to PCR amplification.

The expression of individual gene targets was analyzed using an MX4000 real-time quantitative PCR system (Stratagene, La Jolla) and gene-specific primers as shown in Supplementary Table 2. Each 15 μl DNA amplification reaction contained 10 mM Tris–HCl (pH 8.3 at 20 °C), 50 mM KCl, 3 mM $MgCl_2$, 0.01% Tween 20, 0.8% glycerol, 40,000-fold dilution of SYBR Green I (Molecular Probes Inc., Eugene, OR), 200 μM dNTPs, 83.3 nM ROX reference dye (Stratagene), 10 pmol of each primer (5 pmol for importin), 2 μl of diluted cDNA, and one unit of Platinum Taq DNA polymerase (Invitrogen). The thermocycle program for all gene targets included an initial enzyme activation step at 95 °C (9 min) followed by 40 cycles of 95 °C denaturation (15 s), 55–58 °C annealing (30 s; see Supplementary Table 2), and 72 °C elongation (45 s). Controls lacking cDNA template or Taq DNA polymerase were included to determine the specificity of target cDNA amplification. Quadruplicate reactions were performed for each sample and data were averaged and normalized to expression of the gene encoding the ribosomal protein L8 using the comparative ($\Delta\Delta$) C_t method (http://www.dorak.info/genetics/realtime.html). The integrity of

amplification reactions was confirmed by gel electrophoresis and the presence of a single DNA product. Gene expression data are presented as fold change relative to control animals within the same treatment period.

2.10. Statistics

Tissue-specific gene expression data sets obtained from the biological replicate arrays for each treatment and time point were analyzed for consistency and absolute agreement using intra-class correlation with a two-way random effects model (SPSS Version 12.0, Chicago, IL, USA). The averages of the intra-class correlation coefficients over the replicate sets was not less than 0.78 with most values greater than 0.9 (Supplementary Table 3). Fold change response to each treatment was determined for each gene relative to the control and genes exhibiting fold change values greater than or equal to two were identified. As an additional quality measure and to reduce the chance of making a type I error, we omitted genes which displayed large variability over the replicate sets. For each gene/replicate set, variability was measured using the equation: (maximum value − minimum value)/2 which is appropriate for samples up to 5 per group (Montgomery, 1991). If the variation estimate for the gene/replicate set was greater than 1.1, the gene was removed from the final list of genes affected following chemical treatment for that time point. If less than four observations were available for a given time point and treatment, that gene was also removed from the final list of genes.

Statistical analyses on QPCR data were conducted using SPSS Version 12.0 software (SPSS, Chicago, IL, USA). The non-parametric Kruskal–Wallis one-way analysis of variance was conducted within treatment and tissue data sets. Where $P < 0.05$, pairwise comparisons were done using the Mann–Whitney *U*-test. The tank effect in every treatment concentration was not significant ($P > 0.05$) using the Mann–Whitney *U*-test, therefore, the data of two replicate tanks were combined for further statistical analyses. The mRNA levels of each gene in a given exposure time and chemical concentration/treatment was compared with the respective controls using the Mann–Whitney *U*-test.

3. Results

3.1. cDNA array analyses of gene expression in the hindlimb

We performed cDNA array analysis on hindlimb mRNA transcripts that were isolated 24 h after exposure to T_3 (1.92 nM) or T_4 (10 nM) concentrations that are roughly equivalent in potency (White and Nicoll, 1981; Zhang et al., 2006). The nine gene transcripts that passed the various quality measures and displayed a two-fold or greater change in transcript levels are listed in Table 1. All of the gene transcripts detected were moderately affected between two- and three-fold. With the exception of Co-repressor MITR, all have been previously identified as TH-responsive in anurans (Table 1). The levels of five of these transcripts were affected by T_3 treatment (two increased, three reduced) whereas the abundance of the remaining four were all increased by T_4. Both treatments modulated gene transcripts encoding proteins important in the regulation of transcription. One gene transcript encoding forkhead transcription factor foxd1/brain factor 2 was affected by both treatments (albeit in opposite directions; Table 1). T_4 treatment also increased the levels of two transcripts encoding proteins involved in the regulation of apoptosis whereas T_3 treatment reduced aldolase C mRNA involved in dietary fructose metabolism (Shiokawa et al., 2002).

Exposure to MMI, PTU, and PER was examined at both 24 and 96 h. Most of the genes identified have previously been shown to be responsive to one or more of the chemicals tested (Table 2). Although seven transcripts were affected at each time point, there was no overlap in their identities (Table 2). However, three of the five functional group categories were represented at both time points (structural, transcription control, and transport/binding). PER exposure had the greatest effect on transcript levels compared to MMI and PTU (Table 2).

Table 1
Gene transcripts in the hindlimb that displayed exposure-related changes in levels relative to controls at 24 h

Treatment	Functional group	Gene transcript identity	Genbank accession #	Fold change[a]
1.92 nM T_3	Metabolism	Aldolase C[b]	3928510	−2.1
	Transcription	CCCH zinc finger protein C3H-3[b]	AF061982	3.2
		Co-repressor MITR	Z97214	2.5
		Forkhead transcription factor foxd1/brain factor 2[c]	AF072889	−2.1
		REST co-repressor[b,c]	AF096301	−2.3
10 nM T_4	Apoptosis	Bcl-2-like R11[b]	X82461	2.9
		Neural precursor cell expressed developmentally downregulated gene 8 (NEDD 8)[b,c]	Not submitted	2.4
	Transcription	Myogenic factor myf-5[c]	X56738	2.4
		Forkhead transcription factor foxd1/brain factor 2[c]	AF072889	2.3

[a] Negative values represent fold decrease while positive values represent fold increase.
[b] Previously identified as T_3-responsive in tadpole tissues (Shi and Hayes, 1994; Sachs et al., 2001; Crump et al., 2002; Helbing et al., 2003; Krain and Denver, 2004; Das et al., 2006; Veldhoen et al., 2006a,b,c).
[c] Identified as TH-responsive in the brain (Helbing et al., 2007).

Table 2
Gene transcripts in the hindlimb that displayed changes in levels upon exposure to 100 mg/L methimazole (MMI), 20 mg/L propylthiouracil (PTU), or 4 mg/L perchlorate (PER) relative to controls at the indicated time points

Time point (h)	Functional group	Gene transcript identity	Genbank accession #	Fold change[a]		
				MMI	PTU	PER
24	Cell growth control	Secreted protein, acidic, rich in cysteine (SPARC)[b]	X62483	1.0	−1.2	−2.2
	Hormonal regulation	Thyroid-stimulating hormone β[b,c,d]	L07618	−2.1	−2.1	−2.1
	Structural	51 kDa cytokeratin type I[c]	Y00968	1.0	1.0	11.0
		α-2 collagen type I [b,c]	D88764	2.4	1.6	2.6
	Transcription	Gli2 zinc finger protein[c]	AF109923	1.1	1.0	2.0
		Forkhead transcription factor foxd1/brain factor 2[c,d]	AF072889	1.2	1.3	3.8
	Transport/binding	Importin α1a	L36339	1.0	1.0	14.8
96	Chromatin structure	Cytosine-5-methyltransferase[c,d]	D78638	−1.11	1.3	2.1
	Protein processing	Proteasome subunit Y[c]	D87689	15.8	4.6	13.6
	Signal transduction	Activated protein kinase C receptor RACK1	AF105259	3.0	2.0	1.9
	Structural	Myelin proteolipid protein[c,d]	Z19522	2.1	1.0	1.0
	Transcription	Octamer transcription factor (Oct-1)[c,d]	X17190	1.2	3.0	1.2
		Transcription intermediary factor tif2[c,d]	AJ243119	1.0	1.0	5.5
	Transport/binding	Metallothionein[c,d]	M96729	1.0	2.5	6.6

[a] Negative values represent fold decrease while positive values represent fold increase.
[b] Identified as MMI, PTU, and/or PER-sensitive in the brain (Helbing et al., 2007).
[c] Previously identified as T_3-responsive in tadpole tissues (Shi and Hayes, 1994; Sachs et al., 2001; Crump et al., 2002; Helbing et al., 2003; Krain and Denver, 2004; Das et al., 2006; Veldhoen et al., 2006a,b,c).
[d] Identified as TH-responsive in the brain (Helbing et al., 2007).

3.2. cDNA array analyses of gene expression in the tail

Previous work in the tail of multiple frog species has indicated that a suitable time point for the evaluation of TH-dependent changes in gene expression is 48 h after exposure; well before overt morphological changes are evident (Crump et al., 2002; Veldhoen et al., 2006a,b,c; Veldhoen et al., 2006a,b,c; Veldhoen et al., 2006a,b,c; Zhang et al., 2006). We, therefore, examined tail-specific gene expression profiles following exposure to 1.92 nM T_3 or 10 nM T_4 at this time point. Eleven gene transcripts that passed the various quality measures and displayed a two-fold or greater change in transcript levels are listed in Table 3. The vast majority of the gene transcripts (nine) were affected upon T_3 treatment. These included transcripts encoding two tubulin structural proteins, three factors important in cell growth control (cdc2 kinase homologous protein, cyclin D2, and nerve growth factor), two proteins involved in transcription control, and regulators of apoptosis and signal transduction pathways (Table 3). There was no overlap in identities of the transcripts affected by the two forms of TH (Table 3). However,

Table 3
Gene transcripts in the tail that displayed exposure-related changes in levels relative to controls at 48 h

Treatment	Functional group	Gene transcript identity	Genbank accession #	Fold change[a]
1.92 nM T_3	Apoptosis	Requiem	AB021737	−2.0
	Cell growth control	Cdc2 kinase homologous protein[b,c]	M60680	2.1
		Cyclin D2[c]	X89476	3.6
		Nerve growth factor[b,c]	X55716	−2.1
	Signal transduction	ALK-2 receptor	AF012245	2.3
	Structural	α Tubulin[b]	X07046	4.8
		β Tubulin[b,c]	L06232	3.8
	Transcription	Myb-related protein 1 (myb 1)	M75870	−2.2
		Transcription intermediary factor tif2[b,c]	AJ243119	−3.8
10 nM T_4	Cell growth control	Cyclin B1[b]	J03166	2.5
	Metabolism	Mitochondrial cytochrome c oxidase subunit 1[b]	M10217	2.1

[a] Negative values represent fold decrease while positive values represent fold increase.
[b] Previously identified as T_3-responsive in tadpole tissues (Shi and Hayes, 1994; Sachs et al., 2001; Crump et al., 2002; Helbing et al., 2003; Krain and Denver, 2004; Das et al., 2006; Veldhoen et al., 2006a,b,c).
[c] Identified as TH-responsive in the brain (Helbing et al., 2007).

Table 4
Gene transcripts in the tail that displayed changes in levels upon exposure to 100 mg/L methimazole (MMI), 20 mg/L propylthiouracil (PTU), or 4 mg/L perchlorate (PER) relative to controls at 48 h

Functional group	Gene transcript identity	Genbank accession #	Fold change[a]		
			MMI	PTU	PER
Cell growth control	Cyclin H[b,c]	U20505	1.6	3.5	1.0
Hormonal regulation	Retinoic acid converting enzyme	AF057566	2.4	1.0	1.0
Protein processing	Proteasome subunit Y[c]	D87689	3.2	1.2	1.8
Signal transduction	Phosphoinositide 3 kinase catalytic subunit[d]	AF204924	2.3	1.0	1.0
Structural	Non-muscle tropomyosin (TM-4)[c,d]	L35238	2.8	−1.1	1.4
	Coronin[b,c]	AB025246	2.0	1.1	1.0
Transcription	Forkhead transcription factor foxd1/brain factor 2[c,d]	AF072889	2.6	1.0	−1.1
	NK-2 homeobox homolog[c,d]	S65507	3.1	1.7	1.3
	Transcription intermediary factor tif2[c,d]	AJ243119	5.2	1.3	1.7
	Poly A-binding protein ABP-EF[b,c]	M27072	1.4	4.2	2.6
Transport/binding	Metallothionein[c,d]	M96729	6.3	1.5	−1.7

[a] Negative values represent fold decrease while positive values represent fold increase.
[b] Identified as MMI, PTU, and/or PER-sensitive in the brain (Helbing et al., 2007).
[c] Previously identified as T_3-responsive in tadpole tissues (Shi and Hayes, 1994; Sachs et al., 2001; Crump et al., 2002; Helbing et al., 2003; Krain and Denver, 2004; Das et al., 2006; Veldhoen et al., 2006a,b,c).
[d] Identified as TH-responsive in the brain (Helbing et al., 2007).

one T_4-modulated transcript encoded cyclin B1 which regulates cell division, while a second mRNA encodes a critical regulator of metabolism (Table 3).

In contrast to the hindlimb, exposure to PER had minimal effect on the tail (Table 4), while MMI had the greatest influence on gene expression in this tissue with 9 out of 11 genes of all treatment exposures affected (Table 4). The proteins encoded by these transcripts span multiple functional groups and include cell growth control, hormonal regulation, protein processing, signal transduction, structural, transcription control, and transport/binding. Most of the gene targets identified have previously been shown to be responsive to one or more of the chemicals tested (Tables 3 and 4). Interestingly, the transcript levels of forkhead transcription factor foxd1/brain factor 2 were also affected in the tail as in the hindlimb except that MMI exposure rather than PER elicited a response (compare Tables 2 and 4).

3.3. Identification of gene expression biomarkers using QPCR

We chose a subset of candidate mRNAs identified by cDNA array analysis that included a two-fold or greater change in mRNA abundance relative to the controls (Tables 1–4) to subsequently perform QPCR analyses. In a few situations, we also did QPCR analyses on select gene transcripts that did not pass the stringent quality measures imposed during array analyses, but showed indications of having utility as potential QPCR biomarkers. For example, if there was insufficient good data for a gene transcript for one treatment condition (e.g. due to high background or poor spot quality), that transcript was removed from the final cDNA array results, despite the rest of the data clearly indicating that the transcript responded to a treatment exposure. We focused exclusively on the 48 h time point for tail samples and a combination of 24 and 96 h time points for the hindlimb depending upon the gene transcript.

Expression of metallothionein-encoding transcript in the tail was significantly increased by approximately seven-fold relative to the control animals upon MMI exposure, but not in any other exposure condition (Fig. 1). The hindlimb showed a lesser, but significant, response upon exposure to PER (Fig. 1). There were also indications that MMI and PTU elevated the levels of this transcript, but the observed variation under these exposure conditions precluded any significant changes. As with the tail, none of the T_3 or T_4 exposures showed a significant change in metallothionein transcript levels from the control (Fig. 1).

The hindlimb also showed sensitivity to chemical exposure when examining transcript levels encoding 51 kDa cytokeratin type I. MMI, PTU, and PER all elicited a significant increase in transcript levels relative to the controls at 24 h (Fig. 2) whereas T_3 treatment showed no effect except for an increase in transcript levels at 1.92 nM. Only the highest concentration of T_4 resulted in a significant decrease in cytokeratin mRNA levels at this time point (Fig. 2). At 96 h, the hindlimb did not show any significant change with T_3 treatment and the lowest concentration of T_4 showed a significant increase in cytokeratin transcript levels (Fig. 2). In the tail, this transcript was significantly elevated in PTU and PER exposures and at the lowest concentration of T_3 tested. T_4 treatment did not change cytokeratin transcript levels (Fig. 2).

A transcript encoding importin was found to be markedly induced in the hindlimb at 24 h in the presence of MMI or PER, but not PTU (Fig. 3). Neither hormone treatment significantly affected importin expression in this tissue. Exposure to MMI, PTU, and PER reduced importin mRNA levels in the tail at 48 h (Fig. 3B) whereas T_3 treatment did not affect transcript levels except for a decrease was noted at 1.92 nM and T_4 had no effect (Fig. 3).

Two transcripts that showed a significant change upon exposure to T_4 alone were RNA helicase II/Gu (Fig. 4) and defender against death 1 (DAD1; Fig. 4). Both showed indications of sen-

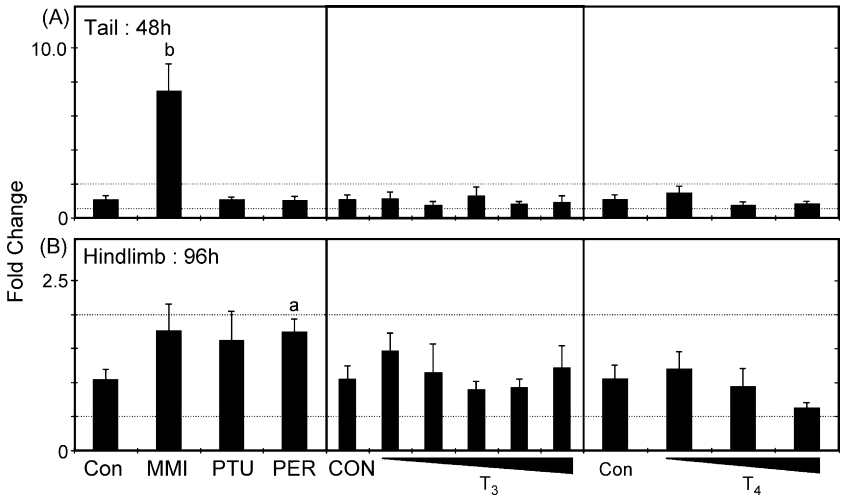

Fig. 1. QPCR analysis of metallothionein transcript levels in the *Xenopus laevis* tadpole tail at 48 h or hindlimb at 96 h. mRNA transcript levels were normalized to ribosomal protein L8 transcript levels and fold changes were calculated relative to time-matched vehicle controls (Con). Error bars represent SEM and those treatments showing a significant change in mRNA levels relative to the controls are indicated by an "a" ($P < 0.05$; MWU) or "b" ($P < 0.01$; MWU). The actual concentrations used for each treatment are indicated in Section 2. The results are the average of 8–10 biological replicates for methimazole (MMI), propylthiouracil (PTU), or perchlorate (PER) and 4 biological replicates for each concentration tested in the T_3 and T_4 sets. Horizontal stippled lines denote two-fold decrease and increase in mRNA levels compared to the controls.

sitivity to PER, but the variation was too great for this mRNA target to serve as a reliable indicator (Fig. 4).

mRNA encoding cyclin D2, a protein important in cell cycle control, was identified by array analysis to be responsive to T_3 treatment in the tail (Table 3). Although a significant increase was also detected using QPCR at the concentration tested for arrays, none of the other concentrations tested showed a significant change relative to controls (Fig. 4).

Most of the gene transcripts that were examined by the QPCR method showed similar trends compared to the array data. How-

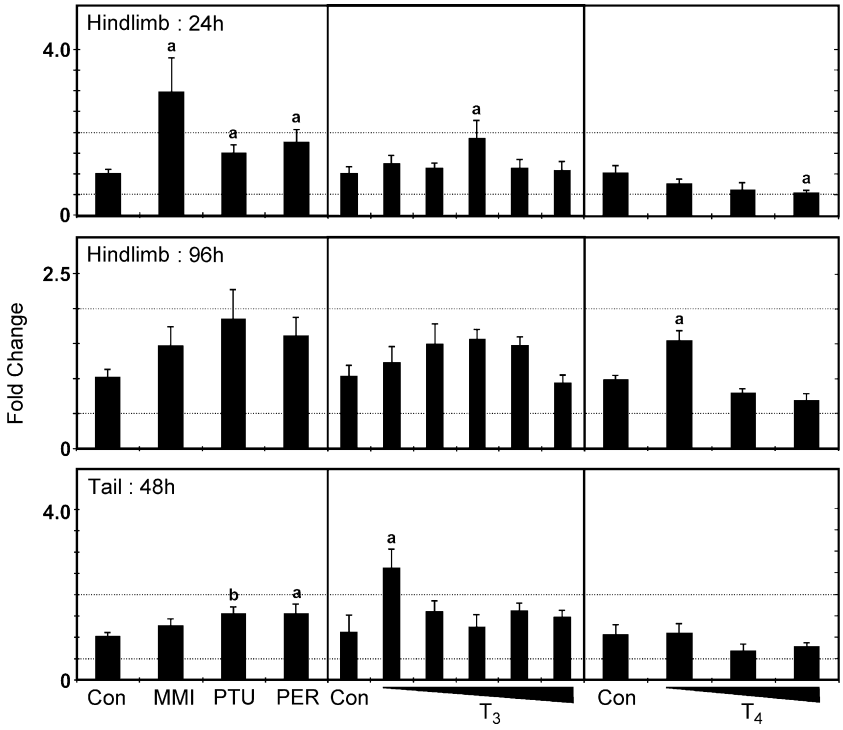

Fig. 2. QPCR analysis of 51 kDa cytokeratin type I transcript levels in the hindlimb of *Xenopus laevis* tadpoles at 24 or 96 h and tail at 48 h. mRNA transcript levels were normalized to ribosomal protein L8 transcript levels and fold changes were calculated relative to time-matched vehicle controls (Con). Error bars represent SEM and those treatments showing a significant change in mRNA levels relative to the controls are indicated by an "a" ($P < 0.05$; MWU) or "b" ($P < 0.01$; MWU). The actual concentrations used for each treatment are indicated in Section 2. The results are the average of 8–10 biological replicates for methimazole (MMI), propylthiouracil (PTU), or perchlorate (PER) and 4 biological replicates for each concentration tested in the T_3 and T_4 sets. Horizontal stippled lines denote two-fold decrease and increase in mRNA levels compared to the controls.

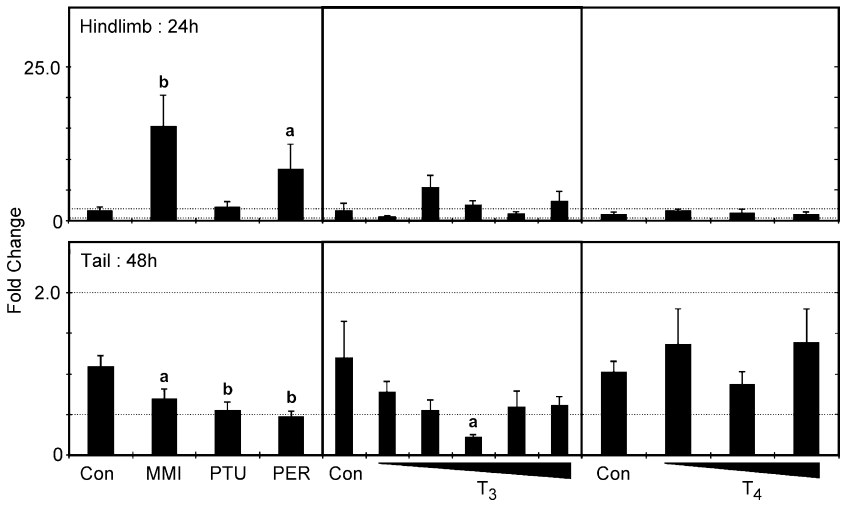

Fig. 3. QPCR analysis of importin transcript levels in the hindlimb of *Xenopus laevis* tadpoles at 24 h or the tail at 48 h. mRNA transcript levels were normalized to ribosomal protein L8 transcript levels and fold changes were calculated relative to time-matched vehicle controls (Con). Error bars represent SEM and those treatments showing a significant change in mRNA levels relative to the controls are indicated by an "a" ($P < 0.05$; MWU) or "b" ($P < 0.01$; MWU). The actual concentrations used for each treatment are indicated in Section 2. The results are the average of 8–10 biological replicates for methimazole (MMI), propylthiouracil (PTU), or perchlorate (PER) and 3–4 biological replicates for each concentration tested in the T_3 and T_4 sets. Horizontal stippled lines denote two-fold decrease and increase in mRNA levels compared to the controls.

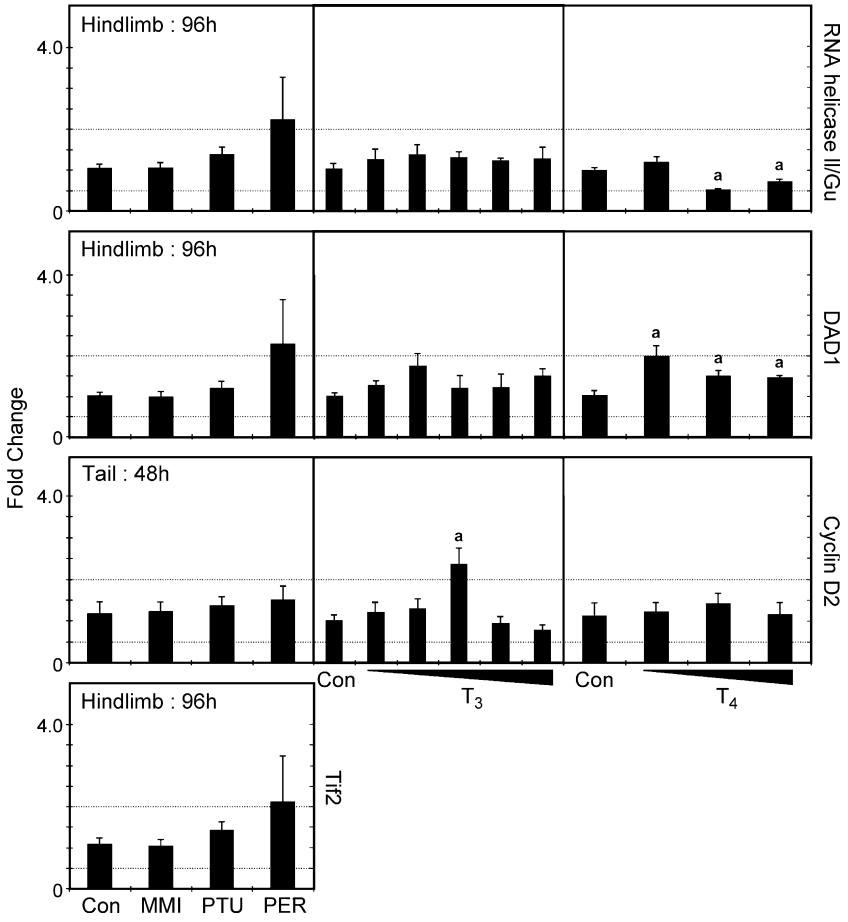

Fig. 4. QPCR analyses of RNA helicase II/Gu and DAD1 in the hindlimb at 96 h, cyclin D2 in the tail at 48 h, and tif2 mRNA transcript levels in the hindlimb at 96 h in the presence of the indicated treatments. mRNA transcript levels were normalized to ribosomal protein L8 transcript levels and fold changes were calculated relative to time-matched vehicle controls (Con). Error bars represent SEM and those treatments showing a significant change in mRNA levels relative to the controls are indicated by an "a" ($P < 0.05$; MWU). The actual concentrations used for each treatment are indicated in Section 2. The results are the average of 8–10 biological replicates for methimazole (MMI), propylthiouracil (PTU), or perchlorate (PER) and 4 biological replicates for each concentration tested in the T_3 and T_4 sets. Horizontal stippled lines denote two-fold decrease and increase in mRNA levels compared to the controls.

ever, many did not yield significant results due to a high degree of biological variation or no significant change with higher numbers of replicates displaying smaller variation (data not shown). For example, tif2 transcripts showed an increase in the hindlimb at 96 h compared to controls (Table 2 and Fig. 4). However, the large variation in the QPCR data set prevented detection of significant changes. Therefore, under the experimental design employed, such a gene transcript would not be suitable for use as a robust biomarker candidate.

4. Discussion

The sensitivity of *X. laevis* tadpoles to thyroid axis disruption makes this species a useful tool for endocrine disruptor screening. We investigated the possibility of identifying gene transcripts that could serve as useful biomarkers in the context of a metamorphosis assay. Using a two-tiered analysis approach, we first performed a cDNA array screen for potential biomarker candidates which assisted in informing us of subsequent gene transcripts to examine in a more quantitative QPCR-based assay. Different tissues respond in different ways to TH and chemical exposure and it is important to define appropriate temporal windows of observation and individual tissue sensitivities in the development of reliable indicators of disruption.

The hindlimb and tail represent two tissues that respond in distinctly different ways to TH action. The former grows and differentiates whereas the latter undergoes apoptosis and regression. These developmental programs require changes in the transcriptome and a subsequent reprogramming of tissue fate. We previously identified the relative sensitivities of these tissues as measured by perturbations in TRα, TRβ, and BTEB expression levels upon exposure to a concentration series of T_3 and T_4, MMI, PTU, and PER (Zhang et al., 2006). All concentrations of chemicals used showed clear acceleration or inhibition of metamorphic parameters within the context of a 14-day metamorphosis assay (Degitz et al., 2005; Tietge et al., 2005; Zhang et al., 2006). Although these three gene transcripts were effective markers for exposure to TH agonists, particularly in the tail, they were insufficient in identifying exposure to TH synthesis inhibitors (Zhang et al., 2006). More extensive analyses presented herein identified novel gene transcripts that could be added to a growing battery of targets. In contrast to the brain (Helbing et al., 2007), considerably fewer gene transcripts were affected by the treatments in either the hindlimb or the tail. This may reflect a reduced sensitivity of the hindlimb to perturbation of TH action at the early prometamorphic stage of development and/or a relative simplicity of the gene expression program initiated for the eventual regression of the tail.

Almost all of the gene transcripts identified in the tail and hindlimb have previously been associated with T_3 induction in other studies at higher concentrations than were tested in the present investigation (Tables 1–4) and a subset of these was also identified in the brain (Helbing et al., 2007). Two particularly striking observations were made with the cDNA array analyses. First, comparison of the mRNA expression profiles observed of T_3 *versus* T_4 exposure reveals that there is very little overlap in response to these two TH agonists. In fact, where there is overlap (e.g. foxd1/brain factor 2 in Table 1), the transcripts displayed opposing changes in abundance that are not predicted by a simple conversion of T_4 to T_3 in the cell or correcting for differences in binding affinities to the TRs (Zhang et al., 2006). This observation also held true in the brain (Helbing et al., 2007) and reinforces the idea that the mechanism of T_4 signaling and subsequent modulation of the transcriptome is not necessarily equivalent to T_3, particularly over the short term (within 96 h). A likely explanation for this includes differential ability for these hormones to induce phosphorylation signaling cascades that influence hormone-inducible gene expression (Bassett et al., 2003). Second, the hindlimb showed a greater sensitivity to PER than the tail whereas the tail was more sensitive to MMI exposure as indicated by the proportion of gene transcripts affected in each at a given time point (Tables 2 and 4). There were no clear-cut subgroupings of affected mRNA based upon chemical modes of action (e.g. between MMI/PTU and PER). These observations reinforce the importance of examining multiple tissues and suggest that the short-term effects observed following chemical exposure were on the tissues directly rather than by perturbation of TH synthesis.

cDNA array analysis has the advantage of being able to ascertain changes in the transcript levels of multiple genes simultaneously which makes this methodology useful in the identification of potential biomarker mRNA species for a given experimental condition. However, determination of the efficacy of individual biomarkers must involve a technique, such as QPCR, that displays greater quantitative ability and is more amenable to larger numbers of biological replicates. Therefore, we used the cDNA array data as a primary biomarker selector for the development of robust QPCR-based assays.

Three gene transcripts, metallothionein, 51 kDa cytokeratin type I, and importin, were identified that showed significant changes upon exposure to one or more of the chemicals: MMI, PTU, or PER. Metallothioneins protect against a variety of stressors including metal toxicity and oxidative stress and also play a structural role in cells (Vasak, 2005). Previous work indicated that metallothionein transcript levels in the tail are responsive following exposure to 100 nM T_3 (Helbing et al., 2003). Interestingly, exposure to lower concentrations of T_3 and T_4, showed no significant effects on this transcript. Therefore, the appearance of this transcript under high-dose conditions may be a reflection of cellular stress rather than a hormonal response *per se.*

Keratins are members of the intermediate filament (IF) protein family important in cell structure. They are also involved in response to stress, apoptosis, and cell signaling (Marceau et al., 2001; Oshima, 2002). Transcript levels of 51 kDa cytokeratin type I decreased in the tail of premetamorphic tadpoles upon exposure to 100 nM T_3 in two independent studies (Crump et al., 2002; Helbing et al., 2003). This decrease was attenuated by the presence of the herbicide acetochlor which alters precocious metamorphosis (Crump et al., 2002). In the present study in which we examined chemical exposure of older, early prometamorphic tadpoles, we did not recapitulate this finding. Rather the lowest T_3 concentration showed an increase in the levels of this transcript (Fig. 2). The difference in results could be due to the amount of hormone administered, the difference between

methods of administration, and/or the developmental stage of animals used in the studies. However, the fact that MMI, PTU, and PER were able to affect transcript levels may compromise its utility as a biomarker.

Importins are karyopherins which are proteins important in the regulation of protein trafficking between cytoplasmic and nuclear compartments (Ziegler and Ghosh, 2005). Their role in the transport of steroid/TH receptors is well-established and constitutes a major step in the regulation of hormone action (Pemberton and Paschal, 2005; Poon and Jans, 2005; Ziegler and Ghosh, 2005). Thus, the alteration in expression of importin mRNA by MMI, PTU, and PER in the hindlimb and tail may represent a novel cellular mechanism of action of these chemicals through alteration of nuclear receptor transport.

We discovered two additional transcripts encoding RNA helicase II/Gu and DAD1 that were decreased and increased, respectively, upon exposure to T_4 in the hindlimb but was unaffected any other exposure condition examined (Fig. 4). RNA helicase II/Gu is involved in the regulation of ribosomal RNA biogenesis. The functional consequence of this result in the hindlimb is not clear, though it may be related to a change in protein translation status of the tissue. This is consistent with the observation that a down-regulation in the oocyte has been linked with the depletion of 18S and 28S RNAs (Yang et al., 2003). DAD1 is involved in the protection of cells from apoptosis (Nakashima et al., 1993).

It is becoming increasingly evident that in order to be able to discern the biological effects of potential endocrine disruptors, multiple endpoints from different tissues at different times should be examined. In the context of a metamorphosis assay, limitations in assay duration and sampling frequency necessitate the establishment of defined assay parameters such as sampling time and tissue source. Therefore, endpoints must be chosen that provide strong predictive value. The present results show that different transcripts display variation in spatial and temporal responses to chemical exposure. In some cases, dose–response relationships are clearly evident such as for TRβ and BTEB transcripts in the tail (Zhang et al., 2006) while in other cases, the relationships are not so clear and may only be detected over a wider temporal scale (for example in Figs. 2 and 3). However, at a given time point and in a select tissue type, we have identified robust responses in gene transcripts that have the potential for incorporation into the *Xenopus* metamorphosis assay through individual or multifactorial analyses (such as correspondence analyses, Helbing et al., 2007). The addition of such endpoints will greatly augment the predictive and diagnostic power of such a screen for the detection of disruptors of TH action.

Acknowledgements

We thank Jeff Ricketson and Rachel C. Skirrow for technical assistance and Dr. Mary Lesperance for her helpful suggestions for statistical analyses. This work was funded by an EPA cooperative agreement to CCH. CCH is a Michael Smith Foundation for Health Research scholar and is recipient of a NSERC University Faculty Award and an early career award for applied ecological research from the Society of Environmental Toxicology and Chemistry. This work was supported in part with funding provided by the American Chemistry Council through a Cooperative Research and Development Agreement (CRADA) with the EPA's Mid Continent Ecology Division. The information in this document has been funded in part by the US Environmental Protection Agency. It has been subjected to review by the National Health and Environmental Effects Research Laboratory and approved for publication. Approval does not signify that the contents reflect the views of the Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2007.02.014.

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